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14. ABSTRACT
The objectives of this project are to quantitatively define the biochemical and cellular mechanisms by which salmon thrombin may be responsible for alleviating pain. Work during the project period utilized biochemical, in vitro, and in vivo approaches to understand and define how human and salmon thrombin differ enzymatically and in modulating cellular inflammatory responses. We established relevant culture systems for making these assessments in astrocytes and mixed cultures and determined that salmon thrombin can reduce astrocytic activation, cytokine production, and not modify cellular mechanics. Substrates were identified for defining cleavage rates of PARs and comparisons between human and salmon thrombin revealed that salmon thrombin cleaves PAR1 at a significantly slower rate than human thrombin does. Our data suggest this may be due the lower affinity for hirudin. PAR1 is decreased in pain states, differentially based on the type of neural trauma and in association with the absence of pain. Early cleavage of PAR1 by thrombin may provide its anti-nociceptive properties. We were very productive, having met all of the milestones that were laid out in the approved statement of work. We have reported findings in presentations and manuscripts and are also poised to continue these investigations having proven our initial hypothesis that salmon thrombin has different properties from other species and that those properties confer tremendous potential for pain relief and tissue healing.

15. SUBJECT TERMS
Enzymatic activity, cell culture, inflammatory response, PARs, thrombin receptor, cleavage rate

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Table of Contents

Page

INTRODUCTION

This project focuses on defining how salmon thrombin can serve as a novel biomaterial to simultaneously reduce pain, while also promote hemostasis and wound healing subsequent to neural trauma. Broadly, the objectives of studies under this project were to quantitatively define the biochemical and cellular mechanisms by which salmon thrombin may be responsible for alleviating pain and to test if, and how, salmon thrombin can achieve a reduction in pain from painful nerve injury. This research project utilizes biochemical, in vitro, and in vivo approaches to define mechanisms of action and to evaluate effects of salmon thrombin on mitigating pain responses. We hypothesized that differences in the catalytic activities between human and salmon thrombin and differences in immune cell activation, make salmon thrombin effective at reducing pain while also promoting wound healing and neuronal survival after neural trauma. Work under this project has focused on measuring rates of proteolysis of thrombin substrates by human and salmon thrombin, quantitatively comparing cellular activation by human and salmon thrombin, measuring cytokine production by mammalian inflammatory cells in response to human and salmon thrombin, quantifying effects of thrombin treatment on cellular mechanics, and evaluating recovery, pain responses and cellular mechanisms with thrombin formulations in an in vivo model of painful nerve trauma. In the first year of this project we made good progress on all studies and met the timeline of activities and milestones that were laid out in the approved statement of work. The details of those efforts were previously summarized in detail in the annual report submitted in the Fall of 2011. In April 2012, we submitted a request for no-cost-extension of the project activities, which was granted in July of 2012. Since our last report of progress, we continued to make substantial progress in all areas, including publishing work from Aims 1 and 3, as well as focusing primarily on the in vivo studies under Aims 4 and 5. During the period of this award, we presented our findings at several national meetings related to basic science, clinical groups and military health services, have applied for funding from other mechanisms using this work as pilot data, and have several publications and several more due to be submitted based on the work completed under this project. We are poised now to continue to investigate our initial hypothesis in more detail and with more specific grounding, based on this Hypothesis Development Award.

BODY

Since our last report we have made substantial progress on all Tasks and have met the milestones proposed in the approved statement of work. We have presented/published our work in a variety of venues and continue to do so. In this portion of the report we include those methods and results in detail that were not previously summarized in our last annual report and that have not been reported or included in other publications. Where applicable, we refer to those publications, abstracts and presentations; the abstracts and presentations are provided in the Appendix. A primary goal of this work was to study how human and fish thrombin differ enzymatically in order to understand why the pain response with treatment from each species is different. As such, coordinated studies using in vitro and in vivo approaches were performed in this project. We structure this section of the report to provide an overall summary of each task, followed by a more-detailed report of the relevant data and findings.

The GANTT chart below summarizes the specific tasks that were associated with each aim across the entire project period under the approved statement of work. Before providing a detailed summary of the research findings, we indicate the current status of each activity to provide an overview of the research activities completed and the remaining activities. For all activities, we have completed the experiments and analysis and the remaining activities are limited to publication in only a few Aims (Milestone #6). Of note, the original Tasks related to the microfluidics for macrophage migration studies (Tasks 3d and 3e) were redirected to focus on defining the activation responses (mechanical and inflammatory) of astrocytes since that work is more-relevant and meaningful given the findings from our studies to date on inflammation and pain [Dong & Winkelstein 2010; Rothman & Winkelstein 2010;

Dong et al. 2013; Smith et al. *submitted*]. This modification was previously addressed in our prior approved report. Further, the macrophage responses are complemented by studies of blood-spinal cord barrier breakdown in the in vivo studies under Aims 4 and 5.

TASK	Q1		Q2		Q3		Q4	
TASK 1 – Aim 1: Proteolysis studies								
1a. Acquire thrombin	completed							
1b. Define protease activation	completed							
1c. Data analysis & integration	completed							
1d. **Milestone #2**–Publish findings	completed							
TASK 2 – Aim 2: Cell activation studies (in vitro)								
2a. Establish cell cultures & test thrombin concentrations		completed						
2b. Perform activation assays		completed						
2c. Data analysis & integration			completed					
2d. **Milestone #4**–Publish findings		Studies completed & *publication in 2013*						
TASK 3 – Aims 3 & 4: Cytokine (Aim 3) & Macrophage migration (Aim 4) studies (in vitro)								
3a. Test thrombin concentrations & optimize time points		completed						
3b. PCR & IHC assays for day 3 & day 7		completed						
3c. PCR & IHC assays for 6 hr & day 1			completed					
3d. Set-up thrombin gradient techniques		removed						
3e. Macrophage migration studies			removed & *modified for in vivo*					
3f. Data analysis & integration				completed				
3g. **Milestone #5**–Publish findings		completed						
TASK 4 – Aims 4 & 5: Behavioral studies (in vivo)								
4a. **Milestone #1**–Obtain regulatory approval for rat studies	completed							
4b. Acquire thrombin					completed			
4c. **Milestone #3**–Identify thrombin concentrations for rat studies			completed					
4d. Perform day 14 rat studies						completed		
4e. Perform day 3 & 7 rat studies							completed	
4f. IHC assays & analysis (rat)						completed		
4g. Analysis of rat data							*ongoing*	
4h. **Milestone #6** – Submit publication (rat studies)			*planned publications in early 2013*					

Task 1

Work under **Task 1** corresponds to Aim 1 which had the main goal of evaluating the proteolysis rate of human and salmon thrombin. Thrombin is a serine protease that cleaves fibrinogen to form fibrin, but it also is responsible for many of the cascades that cause pain and inflammation and stimulates inflammatory cytokine production. Activation of those cascades occurs primarily through the cleavage

of PARs, or protease activated receptors which are G-protein coupled receptors. As detailed in our prior report, there are four PARs –1 ,2, 3 and 4; thrombin can cleave PARs 1, 3 and 4. The PARs are self-activating, so when thrombin cleaves them near the N-terminal the remaining end acts as a tethered ligand that then activates the PAR. There is also a second thrombin receptor site which is a hirudin-like site, which is present on PARs 1 and 3.

In our prior report we summarized activities under **Tasks 1a and 1b** to evaluate if there are enzymatic differences between salmon and human thrombin. The kinetics of protease activation were tested using fluorogenic synthetic substrates that mimic the thrombin receptors. Details of those methods are provided in our publications in the Appendix [Oake et al. 2011; Smith et al. 2012a] and in the paper we recently submitted to *Molecular Pain* [Smith et al. 2012]. For the PAR1 peptide at 37 degrees, human thrombin was found to cleave the peptide faster than fish thrombin. When quantifying the rate of cleavage for each of the PARs tested, it was found that the rate of cleavage for human thrombin is significantly (**p=0.01) faster than for salmon thrombin [Oake et al. 2011; Smith et al. 2012a]. PAR3 and PAR4 were also tested, but the rate of PAR4 cleavage was so slow that any difference was undetectable using this approach; no difference was observed between species in PAR3 cleavage rate [Oake et al. 2011; Smith et al. 2012a].

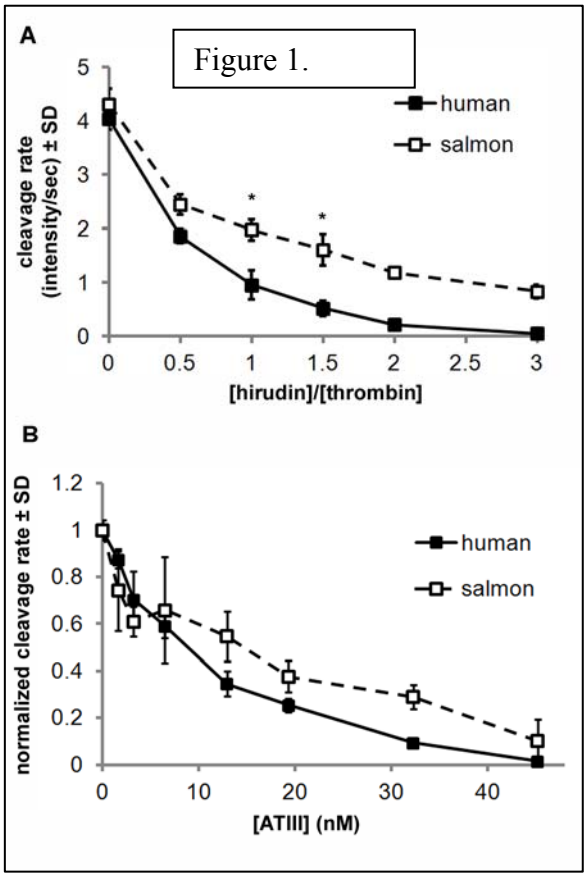


Figure 1.

Thrombin activity, as measured by fibrinogen cleavage rate, was inhibited by hirudin and antithrombin III (ATIII) in separate studies, for both species of thrombin (Figure 1) but with different dose-dependence. Salmon thrombin retains significantly ($p<0.0001$) more activity than human thrombin overall, as evidenced by a faster fibrinogen cleavage rate over a range of [hirudin]/[thrombin] ratios (Figure 1A). Specifically, salmon thrombin exhibits a significantly (*$p<0.002$) faster fibrinogen cleavage rate at ratios of 1 and 1.5 (Figure 1A). In contrast to the differential inhibition by hirudin, there is no difference in the reduction of human and salmon thrombin activities by human antithrombin III (ATIII) (Figure 1B). Taken together, hirudin inhibits salmon less effectively than human thrombin but ATIII inhibited both salmon and human thrombin equally [Smith et al. 2012b].

Synthesizing (**Task 1c**) these collective results we have been able to develop several conclusions and reported (**Task 1d**; **Milestone #2**) them in the 3 presentations (see Appendix) and in 1 submitted manuscript [Smith et al. 2012b] (#2, #3, #6, #8 in Bibliography). Salmon thrombin hydrolyzes peptides mapping the PAR1 cleavage sequence at slower rates than human thrombin, suggesting that salmon thrombin may be less efficient at activating cellular PAR1. This cleavage data suggests there to be an inherent difference in PAR1 cleavage rate between these species that may also be related to the difference in their effectiveness of attenuating pain. In many physiological systems thrombin can initiate dual signaling cascades at least partially based on the type of proteins that are coupled to the activated receptor or the degree of PAR1 activation [Dale & Vergnolle, 2008; Ma & Dorling, 2012]. For example, PAR1 activation by thrombin in endothelial cells can induce vascular protection or vascular leakage depending on which sphingosine 1-phosphate (S1P) receptor PAR1 is coupled to, SIP_1 or SIP_3 [Ma & Dorling, 2012]. Within the nervous system, PAR1 agonist concentration seems to be more important for the subsequent signaling; low concentrations of PAR1-AP decrease levels of phosphorylated ERK

(pERK), a marker of cellular trauma, whereas high concentrations increase pERK expression, suggesting that high concentrations may amplify trauma [Gao et al. 2009; Shavit et al. 2011]. In our studies, salmon thrombin cleaves PAR1-like peptides slower than human thrombin for both the PAR1 cleavage sequence and the cleavage sequence plus a hirudin-like domain [Smith et al. 2012b]. Previous studies show that salmon and human thrombin are equally efficient at cleaving peptides based on the human fibrinogen cleavage site [Michaud et al. 2002]; our findings study agree with prior reports. Since PAR1 is activated slower by salmon thrombin than human thrombin it is possible that salmon thrombin mitigates cellular trauma following nerve root injury while human thrombin may exaggerate trauma.

Although PAR1 activity is different between salmon and human thrombin it is possible that other receptors also are partially responsible for the effects observed here. For example, the activation of thrombin-cleavable PAR4 via a PAR4 activating peptide (PAR4-AP) in cultures of primary sensory neurons attenuates intracellular calcium responses [Asfaha et al. 2007]. It is possible that salmon thrombin activates PAR4 more readily than human thrombin, also contributing to its analgesic properties. In our studies, differences in affinity for various molecules was observed between the two species of thrombin, including the PAR1 cleavage sequence, the PAR1 cleavage sequence with a hirudin-like domain, and the thrombin inhibitor hirudin which suggests that there may also be differences in their affinities for other molecules [Smith et al. 2012b]. Future studies inhibiting the action of the PARs after nerve root injury would provide further information on whether this and other PAR receptors contribute to the analgesic properties of salmon thrombin. Nonetheless, the results from this study indicate that PAR1 activation rate may be a key contributor to the analgesic and anti-inflammatory actions induced by salmon thrombin, which are not exhibited by human thrombin.

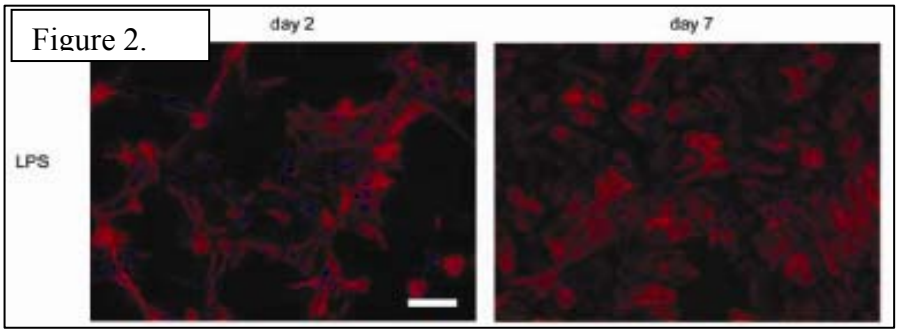
Task 2

Work under **Task 2** corresponds to Aim 2 with the main goal of evaluating if there is a difference in the activation of cells involved in pain in vitro due to stimulation by human and salmon thrombin. We use primary neurons and astrocytes obtained by standard methods from rodents. Under **Task 2a** we established methods for culturing and stimulating isolated astrocytes and mixed cultures of astrocytes and neurons. We evaluated cellular activation in response to thrombin alone and with thrombin treatment following an inflammatory stimulus. Activation was assayed by quantifying changes in cell shape and area and GFAP expression for astrocytes, using routine methods of fluorescence. For proliferating cells, cell counts were performed using DAPI. For both human and salmon, thrombin concentrations were varied between 1U/ml and 10U/ml. Cells were routinely observed for morphologic changes and cytokine production for up to 7 days under these conditions. Based on cytokine production for each concentration, the 10U/ml actually induced increases in IL1β while the 1U/ml resulted in decreased inflammatory response. Further, cells were stimulated using both substance P (a common mediator of nociception) and LPS (a common initiator of inflammation in experimental studies) [Chung & Benveniste 1990; Miyano et al. 2010; Loram et al. 2011]. From those studies that were previously summarized in our prior annual report, it was determined to use a 1U/ml concentration of both human and salmon thrombin in subsequent studies.

Mixed cultures of astrocytes and neurons were used for studies in **Task 2b** and have been previously described in our last report. Cells are plated onto poly-Llysine-treated (PLL; Sigma-Aldrich; St. Louis, MO) T75 tissue culture flasks (Fisher Scientific, Inc.; Pittsburgh, PA) at a concentration of $1x10_5$ cells/ml. Media was changed every 3-4 days. After 13 days *in vitro* (DIV), cells are released from the flasks with trypsin at 37°C. After 5 minutes, enzymatic activity is stopped with serum containing media and cells are centrifuged at 1000 rmp for 5 minutes. Cells are resuspended and plated onto 114 PLL-coated glass bottom dishes (MatTek Corp.; Ashland, MA) at a concentration of $1x10^5$ cells/ml. Cultures are allowed to stabilize at 37°C in 5% CO_2 for 7 days with media changes every 3-4 days prior to any stimulation or treatment. Since in the last report, we summarized our studies of mixed astrocyte/neuron cultures that were stimulated with lipopolysaccharide (LPS) at day 0 at a concentration

of 1 µg/mL, we provide only a brief summary of those findings here. In the mixed culture of astrocytes and neurons, LPS stimulation increases PGE_2 production by approximately 1.2-fold at both day 2 and day 7. LPS stimulation also reduces the astrocyte perimeter by 20% compared to the unstimulated control, indicating activation of astrocytes (Figure 2).

Figure 2.



Based on those studies with LPS, we also administered salmon thrombin at a concentration of 1 µg/ml in mixed neuronal-astrocytic cultures that had been stimulated by LPS and probed for PAR1 expression at 1 hour and 1 day after treatment. PAR1 expression decreases within 1 hour of stimulation, but is returned to control levels when thrombin is given, further supporting the hypothesis that salmon thrombin provides anti-inflammatory effects (Figure 3).

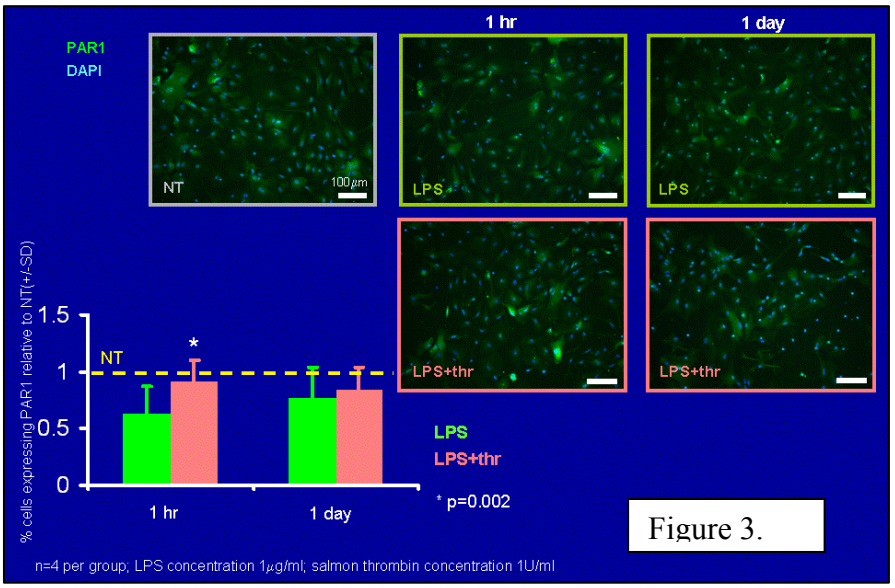
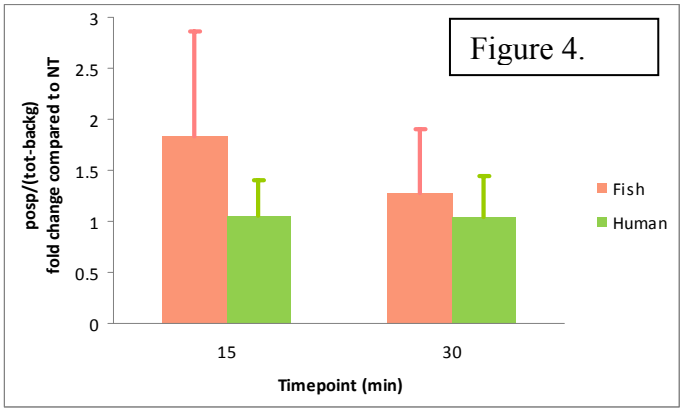


Figure 3.

Extending that work, we evaluated the rate of cleavage of the PAR1 using the in vitro cultures. Human and salmon thrombin were given at the same doses as above and PAR1 expression was assayed at 15 minutes and 30 minutes. The number of cells positive for PAR1 was found to be significantly greater (p=0.001) for salmon thrombin than human thrombin at 15 minutes (Figure 4), supporting the findings that salmon thrombin cleaves the PAR1 N-terminus slower than human thrombin (Task 1; Figure 1). Together, all of these findings were the basis of a larger study that was recently accepted in the *Journal of Neurotrauma* [Dong et al. 2013; #7 in Bibliography].

Figure 4.



In order to extend work defining cell activation, we also implement atomic force microscopy (AFM) to define the cellular mechanics of astrocytes in response to thrombin treatment. The experimental conditions were the same as those described above, with astrocyte cultures treated for 30 minutes with thrombin (either salmon or human) and then rinsed 3 times with PBS. There is mounting evidence that as astrocytes become activated their stiffness is also altered; AFM enables the measurement of cellular modulus. We determined that there is a significant difference (p=0.01) in the stiffness of astrocytes treated with human thrombin compared to untreated controls (Figure 5), while treatment with salmon thrombin does not modify cellular stiffness. These findings are indeed quite exciting as they may provide an explanation for some of the in vivo results we observe under **Task 4**.

As we proceed with analyzing these in vitro studies and the in vivo studies under **Task 4g**, we expect to incorporate these AFM results in the manuscript we are preparing for submission in early 2013 (#9 in Bibliography)

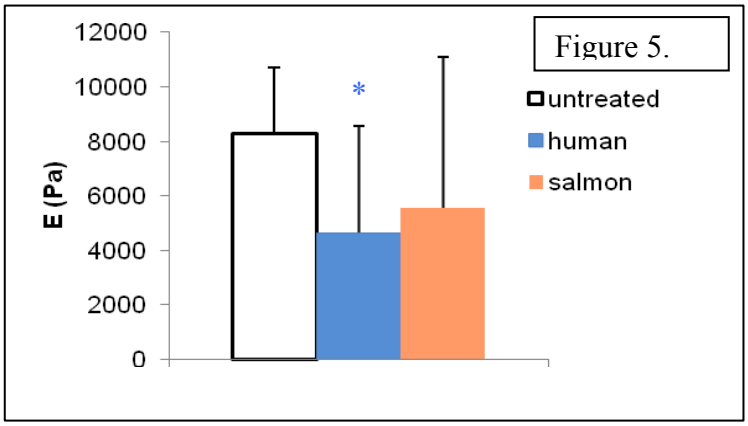


Figure 5.

Task 2c includes the data analysis and integration across Tasks in this Aim 1, and as described above is almost complete. **Task 2d** involves the publication of this work and is ***expected in the next few months***. Parts of these findings have been published and presented in several podium presentations and submitted papers (see Appendix; #6, #7, #8 in Bibliography).

Task 3

Work under **Task 3** corresponds to Aims 3 and 4 of the proposal and focused on quantifying cytokine production in response to thrombin stimulation, at early time points in the in vitro studies of Aim 2. Cytokine production in astrocytes was measured in response to human and salmon thrombin using PCR to define message levels and ELISA to quantify inflammatory mediators implicated in pain. We evaluated TNFα and IL1β because of their known roles as pro-inflammatory agents, and their ability to induce pain [DeLeo & Yezierski 2001]. Under **Task 3a**, we had proposed to establish the cultures and optimize the proposed time points for assessment. We originally proposed to probe responses at 6 hours, day 1, day 3, day 7 after stimulation, but following pilot studies, we in our last report we revised those assessment points to 1 and 24 hours since the cytokine cascade is regulated early and the in vivo studies in Aim 5 will capture the later responses. Accordingly, under **Tasks 3b and 3c**, we performed PCR and ELISA at these time points. In our last report, we detailed findings from studies in which cells were stimulated by 100nM substance P and treated 1 day later with 1U/mL of either human or fish thrombin. Briefly, the amount of IL-1β in the supernatants from the astrocyte cultures was lower (to nearly one-third) for treatment with the fish thrombin compared to the human thrombin ($p=0.037$). However, after 1 day in culture, the fish thrombin was also less active than the human thrombin ($p=0.0003$). These data suggest that the reduced astrocytic inflammatory response to fish thrombin may be caused by the reduced activity of fish thrombin or by the slower proteolysis rate of fish thrombin on PAR1. Those data were included in the publication at the *Annual Meeting of the Biomedical Engineering Society* (BMES) in October 2011 [Oake et al. 2011] (see Appendix). This same significant relationship was observed in cultures of astrocytes alone and in mixed astrocyte-neuronal cultures. Additional studies were performed to assess PAR1 mRNA in spinal cells at two early time points following painful nerve root trauma. The specific details of those methods are included in the abstract that presented this work at the *American Society of Mechanical Engineers Summer Bioengineering Conference* in June 2011 (ASME-SBC) [Smith et al. 2011b] (see Appendix). In summary, those data indicate that decreases in PAR1 mRNA relate to pain and can be regulated early on following the injury. Upon further investigation, we also were able to localize PAR1 expression in both neurons and astrocytes in our cultures (Figure 6). Since that time, we expanded the group sizes and reported that work at the *Military Health System Research Symposium* in August 2012 (see Appendix; #4 in Bibliography).

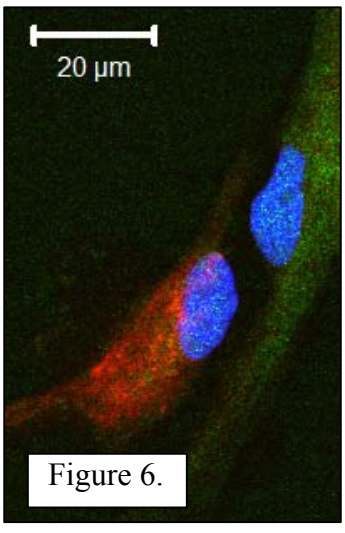
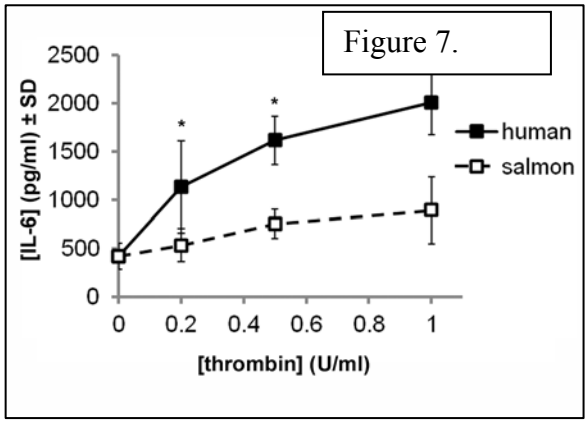


Figure 6.

We also performed additional dose resposne studies with thrombin treatment at different doses to evaluate effects on IL-6 production (Figure 7) [Smith et al. *submitted*]. The concentration of IL-6 protein

in the supernatants of mixed cortical cultures was significantly ($p<0.0001$) increased when the cultures were treated with human thrombin as compared to salmon thrombin (Figure 7). Further, cultures treated with human thrombin released significantly ($p<0.0363$) more IL-6 than cultures treated with salmon thrombin at each individual thrombin concentration of 0.2, 0.5 and 1 U/ml (Figure 7).



Assessments of macrophage migration were originally proposed in studies under **Tasks 3d and 3e**. Of note, we elected to evaluate integrity of the blood brain barrier in the in vivo studies as a more relevant metric of cellular migration since that will provide a more comprehensive understanding of the consequences of such changes in vivo and in the context of pain behaviors. These studies (**Task 3e**) are included in work under Task 4, accordingly. Also, **Task 3f** was removed as per the review detailed in our prior report. Since the main goal is to evaluate these responses in the context of pain such in vivo assessments will provide more added value than simple migration assays in vitro.

Tasks 3f and 3g include the data analysis and integration as well as the publication of this work and is **completed** as described above. To date, parts of the findings under this Aim were published and presented at several meetings and in already submitted and planned manuscripts (see Appendix; #4, #6, #8, #9, #10 of Bibliography).

Task 4

Work under **Task 4** corresponds to Aims 4 and 5 which utilizes an in vivo model of traumatic nerve injury in the rat. Specific sub-tasks of those Aims were the primary effort over the last period of this project. As previously reported, **Tasks 4a-4c** were completed during the prior reporting period and **Tasks 4d-4f** have been ongoing in the last period. We obtained regulatory approval from both the University of Pennsylvania and USAMRMC, in August 2010 and September 2010, respectively (see Appendix for approval letter from Penn IACUC). In addition, it was determined based on the in vitro studies, that thrombin would be used at a concentration of 1U/ml for all in vivo studies (**Task 4c**).

Rats underwent a transient painful compression of the right C7 dorsal nerve root [Smith et al. 2012b; Syre et al. 2012]. Briefly, surgical procedures were performed under inhalation anesthesia with the rat in the prone position (4% isoflurane for induction, 2% for maintenance). An incision was made from the base of the skull to the T2 spinous process. A hemilaminectomy and partial facetectomy were performed on the right side of the C6/C7 spinal levels in order to expose the right C7 dorsal nerve root. The nerve root was compressed for 15 minutes with a calibrated 10 gram-force microvascular clip (World Precision Instruments, Sarasota, FL). Following clip removal, any blood was cleared from the compressed nerve root and 20 µl of either salmon (salmon, n=6) or human (human, n=6) thrombin (2 U/ml in neurobasal media) was added to the nerve root. A separate control group received a vehicle treatment (vehicle, n=6) of 20 µl of only the neurobasal media. Wounds were closed with polyester suture and surgical staples. Rats were allowed to recover in room air under continuous monitoring.

Behavioral sensitivity was assessed in the forepaw by measuring mechanical allodynia on days 1, 3, 5 and 7 post-injury. Allodynia was also measured for each rat before any surgical procedures to establish baseline responses. Prior to each testing session rats were placed in elevated cages with mesh bottoms and allowed to acclimate for 15 minutes. Mechanical allodynia was measured by stimulating the plantar surface of the forepaw on the side ipsilateral to the root compression, using 1.4 and 4 gm von Frey filaments (Stoelting Co., Wood Dale, IL). Testing sessions consisted of three rounds of 10 stimulations to each paw, separated by a 10 minute rest period. A positive response was considered as a paw withdrawal and was often accompanied by licking or shaking of the paw. The number of paw

withdrawals in a session were counted for each rat and averaged within groups for each day. A repeated measures analysis of variance (ANOVA) with Tukey's test was used to determine statistical differences between groups overall and on individual days for each testing filament.

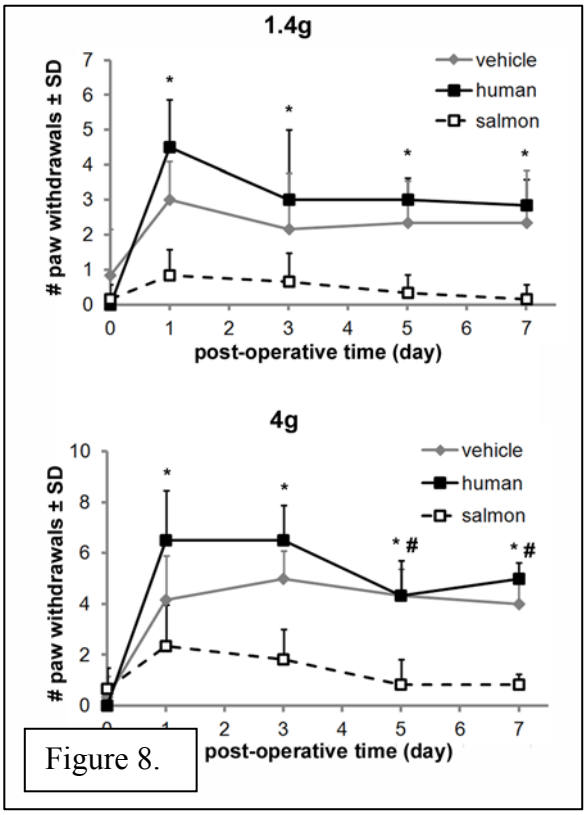


Figure 8.

Mechanical allodynia in the ipsilateral forepaw of rats that received a painful nerve root compression with vehicle treatment of neurobasal media is significantly elevated over baseline responses on all days for testing with the 4 g von Frey filament ($p<0.0001$) and at day 1 using the 1.4 g filament ($p=0.006$) (Figure 8). A single administration of salmon thrombin is sufficient to significantly reduce ($p=0.004$ for 1.4 filament; $p=0.0004$ for 4 filament) allodynia compared to *vehicle* for both von Frey filament strengths. Further, allodynia responses are significantly ($p<0.002$) different between *salmon* and *vehicle* treatments on days 5 and 7 when testing with a 4 g von Frey filament (Figure 8). In contrast, administration of human thrombin does not modify mechanical allodynia after injury on any post-operative day compared to *vehicle* (Figure 8). Rats treated with human thrombin exhibit mechanical allodynia that is significantly elevated over the salmon thrombin group overall ($p<0.0004$) and on each post-operative day ($p<0.036$).

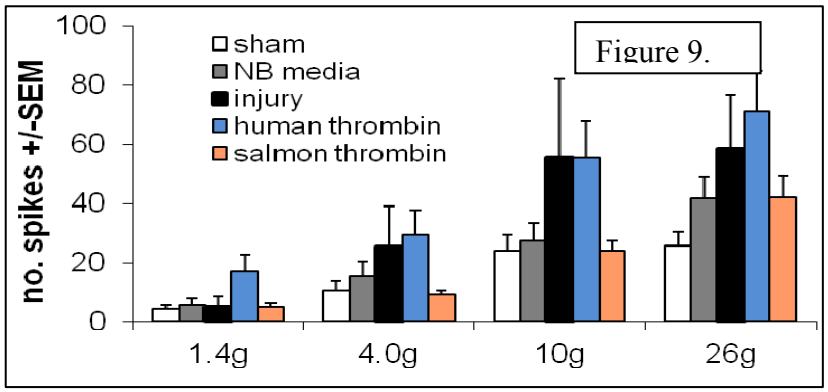


Figure 9.

Additional studies using separate rats (n=4 each group) measured neuronal activity in the spinal cord (Figure 9) and suggest a reduction also in neuronal activity following salmon thrombin treatment. We continue to analyze those data, together with the immunohistochemical data from these same studies for insight into the mechanisms by which salmon thrombin attenuates pain. Although these data were recently presented at 2 scientific meetings [Syre et al. 2012; Smith et al 2012a] (see Appendix; #5, #6 in Bibliography), the in-depth analyses (under **Task 4g**) are ongoing since there is a large amount of data generated. We anticipate submitting 2 more manuscripts from these analyses in early 2013 (**Task 4h; Milestone #6**). Further, these pilot data from early in vivo studies under this project formed the basis of a proposal for continued funding from the *Cervical Spine Research Society*, that we were successful in obtaining funding (see Funding Applied For below).

KEY RESEARCH ACCOMPLISHMENTS

- Established relevant culture systems for assaying thrombin effects on inflammation.
- Determined that both salmon and human thrombin decrease TNFα and IL1β mRNA, but salmon thrombin produces a more robust and significant decrease in astrocytes than does human thrombin.

- Established methodology and identified relevant substrates for studying enzymatic activities of PARs.
- Determined that salmon thrombin cleaves PAR1 at a slower rate than human thrombin.
- Identified that the slower rate of cleavage by salmon thrombin may be due to its lower affinity for hirudin.
- Determination that PAR1 is associated with pain and neural trauma and appears to be regulated in activated immune cells in response to painful trauma.
- Identification that the modifications in PAR1 occur very early following its exposure to thrombin.
- Determined that salmon thrombin does not modify astrocyte cell stiffness whereas human thrombin does.
- Anti-inflammatory treatment that attenuates pain also modulates PAR1 expression in vivo.
- Determination that salmon thrombin given at the time of injury eliminates the development of pain in vivo, in association with attenuating spinal hypersensitivity.

REPORTABLE OUTCOMES

Bibliography of Published Manuscripts & Abstracts (see Appendix for Abstracts & Slides of Presentations)

1. Smith JR, Rothman SM, Janmey PA, Winkelstein BA. Spinal PAR1 mRNA Levels are Regulated by Mechanical & Chemical Cues in Painful Nerve Root Compression. *ASME Summer Bioengineering Conference*, #SBC2011-53084, Nemacolin, PA, June 2011.
2. Oake SA, Smith JR, Janmey PA, Winkelstein BA. Distinct Effects of Human and Salmon Thrombin on the Inflammatory Response of Mammalian Astrocytes. *BMES Annual Meeting*, #Sat-1-2-C, Hartford, CT, October 2011.
3. Smith J, Rothman S, Black J, Winkelstein BA. Spinal PAR1 mRNA Expression Decreases Early After Painful Nerve Root Injury with Inflammation. *BMES Annual Meeting*, #Sat-1-5-A, Hartford, CT, October 2011.
4. Smith JR, Weisshaar CL, Janmey PJ, Winkelstein BA. Salmon Thrombin Treatment Reduces Protease Activated Receptor 1 Expression Following Painful Nerve Root Injury. *Military Health System Research Symposium*, #12-141, Ft. Lauderdale, FL, August 2012.
5. Syré P, Smith JR, Nicholson KJ, Welch WC, Janmey PA, Winkelstein BA. Salmon Thrombin Leads to Decreased Spinal Cord Responsiveness in Painful Radiculopathy. *24th Annual Pan Philadelphia Neurosurgery Conference*, Philadelphia, PA, December 2012.
6. Smith JR, Nicholson KJ, Syré P, Janmey PA, Winkelstein BA. A Novel Bioengineered Biomaterial to Treat Painful Neural Trauma via Modified Thrombin Activity for Improving Neuronal Function and Treating Pain. *Cervical Spine Research Society Annual Meeting*, Chicago, IL, December 2012.
7. Dong L, Smith JR, Winkelstein BA. Ketorolac reduces spinal astrocytic activation and PAR1 expression associated with attenuation of pain following facet joint injury. *Journal of Neurotrauma*, to be published 2013.
8. Smith JR, Oake S, Weisshaar CL, Cruz K, Bucki R, Baumann B, Janmey PA, Winkelstein BA. Salmon and human thrombin differentially regulate radicular pain and inflammation through differences in their rate of cleavage of the protease-activated receptor-1. *Molecular Pain*, submitted.

9. Smith JR, Syré P, Nicholson KJ, Janmey PA, Winkelstein BA. Salmon thrombin attenuates spinal hypersensitivity in association with reduced inflammation and pain following nerve root injury, *to be submitted Spring 2013*.
10. Smith JR, Janmey PA, Winkelstein BA. Blood-spinal cord barrier response after neuropathy is modulated by salmon thrombin in association with attenuation of pain and neural repair, *to be submitted Spring 2013*.

Funding Applied for Based on Work by this Award

1. Jenell Smith, graduate student on this project applied for and received a Student Travel Grant Award from GAPSA at Penn to present the poster presentation for #1 above, 2011.
2. Collaborative Research Grant from Comprehensive Neuroscience Center at Penn in 2011 – *not funded*.
3. 21st Century Grant Award from Cervical Spine Research Society – *funding provided ($75,000)* to implement additional electrophysiology assessments in vivo with thrombin treatment to investigate the neuronal functional responses as follow-on funding in 2012.
4. NIH grant application planned for submission Feburary 2013, using the data in this report as pilot data for that application.

List of Personnel Receiving Pay from this Award

1. Dr. Beth Winkelstein
2. Dr. Paul Janmey
3. Dr. Raz-Ben Arouch
4. Ms. Jenell Smith

CONCLUSION

Salmon thrombin as a biomaterial has a long shelf-life and can be easily deployed in wounds with little-to-no medical expertise. Considering these advantages, together with the results of the studies completed already, salmon thrombin has tremendous promise for rapid translation to provide major benefit for alleviating pain. We **hypothesized** that differences in the catalytic activities between human and salmon thrombin, and differences in the spectra of cell types that are activated by this protein, render salmon thrombin effective at reducing chronic pain. **Studies completed under this award support our original hypothesis and have importance in moving forward**. Among the major findings of importance include the fact that salmon thrombin decreases mRNA and protein for two pro-inflammatory cytokines involved in pain, TNFα and IL1β, more robustly than human thrombin does in astrocytes, which are known regulators of pain. A second major important finding is that salmon thrombin cleaves PAR1 at a slower rate than human thrombin and that this thrombin receptor is also associated with pain. Also, the lower affinity for hirudin that was found for salmon thrombin compared to human may explain the slower cleavage rate for salmon thrombin. Most importantly, perhaps, are the findings from the in vivo pain model. Salmon thrombin treatment reduces pain behaviors, spinal hypersensitivity, inflammation, and PAR1 expression. ***These findings are quite novel and have tremendous implications for both hemostasis and pain.*** In addition, they establish a strong and exciting foundation for future in vivo and in vitro studies to better define the specific cellular and biochemical mechanisms responsible for attenuating inflammation, neuronal injury and pain.

We made only minor changes to our Work Plan from the original proposal, as approved in our last annual report. The collection of other studies (both in vitro and in vivo) removed the need for the macrophage migration studies originally proposed under Tasks 3d and 3e. We believe such an undertaking would indeed have been unnecessary and would have detracted from the exciting in vivo experiments that were carried out under our no-cost-extension of this project. We have evaluated the macrophage response in our studies using salmon fibrin treatment and found them to be highly variable [Weisshaar et al. 2011]. Under this project, we have been very productive, having produced 10 publications and/or abstracts and have successfully received additional follow-on funding and plan to apply for more, based on the promising data that were obtained while evaluating our hypothesis.

Current methods to alleviate pain from neural trauma are limited in effectiveness, are sedative, and are not easy-to-use in combat field conditions. Accordingly, there is a tremendous and immediate necessity for the development of novel approaches to treat trauma injuries that enable pain management and can provide early treatment at the point and time of injury. Findings to date on this project indicate that this product – a salmon thrombin biomaterial – provides very rapid (within 15 minutes) regulation of the cascades that are involved in clotting and that it also mediates inflammatory and possibly nociceptive processes. In addition, the new knowledge regarding the cleavage rates and hirudin binding differences that we have uncovered between these two species can have far-reaching basic science implications as well. This material product has a long shelf-life and can be easily deployed in wounds with little-to-no medical expertise. Taking that information together with the findings from our research under this award, this biomaterial has tremendous promise for rapid translation to provide major benefit to the military should these studies show promise for alleviating pain. We are very encouraged by the findings in this project and are excited to continue to pursue studies further defining the anti-inflammatory and pro-survival pathways of salmon thrombin for pain relief and neural tissue healing.

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APPENDIX

Oake SA, Smith JR, Janmey PA, Winkelstein BA. Distinct Effects of Human and Salmon Thrombin on the Inflammatory Response of Mammalian Astrocytes. *BMES Annual Meeting*, #Sat-1-2-C, Hartford, CT, October 2011.

Distinct Effects of Human and Salmon Thrombin on the Inflammatory Response of Mammalian Astrocytes

S. A. Oake[1], J. R. Smith[1], P. A. Janmey[1], and B. A. Winkelstein[1]
[1]University of Pennsylvania, Philadelphia, PA

Introduction: Thrombin inhibition has the potential to prevent the neurotoxic responses that can be caused by local increases in clotting factors after traumatic neural tissue damage [1,2]. Thrombin triggers cellular responses by activating protease activated receptors (PARs), of which thrombin can cleave three of the four identified subtypes (PAR 1, 3, 4). Thrombin cleavage of PAR 1 and PAR 3 is strongly aided by the hirudin-like sequence of the extracellularly exposed N-terminal [3]. Thrombin activation through these PARs in glia, including astrocytes, propagates inflammation and can lead to pain [1,4]. However, blocking the activity of endogenous thrombin can lead to uncontrolled bleeding which makes it also desirable to reduce the inflammatory effects of thrombin while maintaining its normal functions in coagulation. The effects of thrombin on coagulation, inflammation, and pain evolved separately and engage different substrates. Coagulation is strongly preserved between mammals and fish; the reaction of human coagulation proteins with human and salmon thrombin are nearly indistinguishable [5,6]. In contrast, mechanisms of inflammation and nociception are highly divergent between mammals and fish [6], suggesting that salmon thrombin might have distinct effects on mammalian inflammation. This study compared the activity of human and fish thrombin by measuring the proteolysis rates of PARs, the inhibition of thrombin by hirudin, and effects of thrombin treatment on the inflammatory response of astrocytes.

Materials and Methods: To quantify proteolysis rates, the indicated fluorogenic peptide and thrombin were mixed and the change of fluorescence intensity, which corresponds to cleavage, was recorded. Human and fish thrombin activities were first made equal by normalizing the cleavage rate of a peptide sequence corresponding to fibrinogen. Peptide sequences corresponding to the thrombin cleavage sites in PAR 1, 3 and 4 were functionalized with -AMC fluorogenic groups (Abgent). Hirudin binding affinity to thrombin was quantified by measuring cleavage of the fibrinogen-based substrate after addition of varying ratios of hirudin to thrombin. *In vitro* studies used primary astrocytes harvested from Sprague-Dawley rat pup brains (E18) and prepped for culture (IACUC-approved). After 14 days in culture, cells were stimulated by 100 nM Substance P and treated 1 day later with 1U/mL of either human or fish thrombin. Supernatants were collected 1 day later and IL-1β concentration was quantified using ELISA. Thrombin activity in those supernatants was also quantified using the fibrinogen peptide. Differences between human and fish thrombin were compared using t-tests.

Results and Discussion: At physiological temperature (37°C), fish thrombin cleaves PAR1 approximately 3 times slower than human thrombin ($p=0.0013$). Cleavage rates of PAR3 and PAR4 were not different. Fish thrombin binds hirudin half as strongly as human thrombin at a hirudin/thrombin ratio of 1 ($p=0.008$) and one-third as well at a ratio of 1.5 ($p=0.011$) (Figure 1). In addition, the amount of IL-1β in the supernatants from the astrocyte cultures was also lower (to nearly one-third) for treatment with the fish thrombin compared to the human thrombin ($p=0.037$). However, after 1 day in culture, the fish thrombin was also less active than the human thrombin ($p=0.0003$). These data suggest that the reduced astrocytic inflammatory response to fish thrombin may be caused by the reduced activity of fish thrombin or by the slower proteolysis rate of fish thrombin on PAR1. The slower PAR1 proteolysis rate may be partially explained by fish thrombin's lower affinity for hirudin compared to human thrombin, since the hirudin-like sequence of PAR1 is largely related to its activation [3].

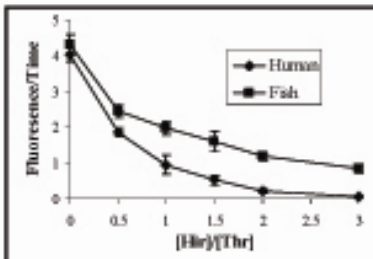

Figure 1. Cleavage of fibrinogen (fluorescence/time) by fish thrombin is significantly less strongly impaired by hirudin than human thrombin at [Hir]/[Thr] ratios of 1 and 1.5.

Conclusions: The reduced inflammatory response by cultured astrocytes to fish thrombin compared to human thrombin may be related to the lower proteolysis rate of PAR1 by fish thrombin compared to human. Future work is needed to quantify PAR cleavage in vitro and define the time course of these modifications as related to inflammation.

Acknowledgements: Support from a DOD grant (W81XWH-10-1-1002) and an Ashton Fellowship.

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Spinal PAR1 mRNA expression decreases early after painful nerve root injury with inflammation

J. Smith[1], S. Rothman[1], J. Black[1], and B. A. Winkelstein[1]
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Introduction: Neural trauma not only induces tissue damage and bleeding but also initiates a local inflammatory response for repair. High concentrations of clotting factors, such as thrombin, are present to promote healing. Thrombin cleaves protease targets including the protease activated receptors (PARs) which leads to the release of clotting and inflammatory factors [1]. PAR activation in platelets is well defined, but only recently has the expression of the PAR subtypes been confirmed for spinal neurons and glia [2,3]. Activation of thrombin-cleaved spinal PARs by injection of thrombin induces both thermal and tactile sensitivity, implicating the role of these receptors in pain [4,5]. Although there is growing evidence that PARs are involved in pain, it is not known whether the spinal expression of these receptors is affected by painful neural injury. The objective of this study was to measure PAR1 and PAR4 mRNA levels in the spinal cord at early time points after two different painful nerve root injuries involving neural compression and inflammation.

Materials and Methods: Separate groups of Holtzman rats underwent a painful C7 nerve root injury [6,7] (IACUC approved): compression (*10gf*, n=11), combined compression and inflammation (*10gf+chr*, n=14), or sham control (sham, n=4). Mechanical allodynia in the ipsilateral forepaw was measured to evaluate behavioral sensitivity (i.e. pain) before surgery (baseline) and at day 1 after surgery. Responses were compared between groups using a repeated-measures ANOVA with Bonferroni. Spinal cord tissue was harvested from separate groups at 1 hour (*10gf* n=8; *10gf+chr* n=9) and day 1 (*10gf* n=3; *10gf+chr* n=5; sham n=4) to assay PAR1 and PAR4 mRNA. Total RNA was isolated for RT-PCR analysis. PAR1 and PAR4 mRNA expression was quantified, normalized by Cyclophilin-A levels, and further normalized to levels in normal unoperated rats. Differences between groups were evaluated for each gene separately using a two-way ANOVA with Bonferroni correction.

Results and Discussion: Both types of compression injuries produced mechanical allodynia in the ipsilateral forepaw at day 1 that was significantly elevated ($p<0.0001$) over their corresponding baseline responses. Yet, only the *10gf+chr* injury produced allodynia that was significantly ($p=0.037$) elevated over sham at that time point. Spinal PAR1 and PAR4 mRNA levels at 1 hour after either injury were unchanged from normal levels. PAR4 mRNA was also unchanged at 1 day after both injuries. In contrast, PAR1 mRNA was significantly reduced ($p=0.0001$) at day 1 after a *10gf+chr* compared to corresponding levels at 1 hour (Figure 1). Spinal PAR1 mRNA was unchanged between time points after the *10gf* injury (Figure 1). These findings imply that spinal PAR1 is modulated within 1 day after a painful injury and may require an inflammatory component for such regulation. Taken together with the allodynia responses, this change in PAR1 in the spinal cord may relate to pain. The decrease in spinal PAR1 transcription does not support reports of its increase at 1 day after spinal cord injury. However, the spinal glial response after spinal cord injury is different temporally than for this injury [7,8].

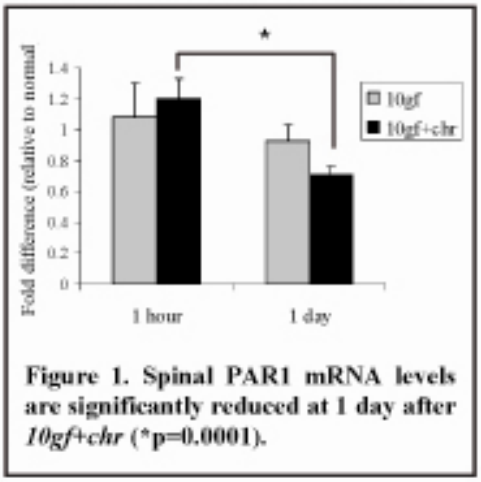

Figure 1. Spinal PAR1 mRNA levels are significantly reduced at 1 day after *10gf+chr* (*p=0.0001).

Conclusions: Spinal PAR1 production decreases early (within 1 day) after nerve root injury with a combined compressive and inflammatory component and this decrease corresponds to the onset of pain. Further studies that quantify this and other PARs at later time points will help to establish their relationship to and role in maintaining pain after neural injury.

Acknowledgements: Support provided by a DOD grant (W81XWH-10-1-1002) and an Ashton Fellowship.

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INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)
(Multiple Project Assurance # A3079-01)

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10-Aug-2010

PRINCIPAL INVESTIGATOR	: BETH A WINKELSTEIN
PROTOCOL TITLE	: Salmon Thrombin as a Treatment to Attenuate Acute Pain and Promote Tissue Healing by Modulating Local Inflammation
GRANT TITLE	: Salmon Thrombin as a Treatment to Attenuate Acute Pain and Promote Tissue Healing by Modulating Local Inflammation
SPONSORING AGENCY	: DEPARTMENT OF DEFENSE
PROTOCOL #	: 803216

Dear DR. WINKELSTEIN:

With receipt of the requested revisions for the above protocol your study now stands fully approved as of **09-Aug-2010**. Work may begin at any time. This study will be due for review on or before **09-Aug-2013**. Protocols are only valid for three years from the date of approval. Please use **Ben Reports (https://galaxy.isc-seo.upenn.edu/ws/benreports)** on a routine basis to check the status of your protocols.

If notification of IACUC review is required by the funding source required, please notify our office in writing of the contact person, agency name, address, phone number, fax number, and email as soon as possible.

Please take note of the following information:

Personnel Training: It is the responsibility of the Principal Investigator to ensure that all persons have completed all necessary IACUC and EHRS training prior to participating in the research described in this protocol.

Amendments*: If you wish to change any aspect of this study, such as procedures, sponsor, analgesics, anesthetics, or the investigators, please communicate your requested changes in writing to the Director for Regulatory Affairs. The new procedures cannot be initiated until Committee approval has been given.

Reapproval*: It is the investigator's responsibility to apply for reapproval of ongoing research annually for protocols involving USDA covered species, or more often if required by the funding agency.

*Forms for amendments and re-approval (Form B) are available from the Office of Regulatory Affairs web site [http://www.upenn.edu/regulatoryaffairs].

Completion of Study: Please notify the Director for Regulatory Affairs as soon as the research has been completed.

Thank you for your cooperation with the Committee.

Sincerely,

Troy Hallman, MS, VMD
Director of Animal Welfare, IACUC

Smith JR, Rothman SM, Janmey PA, Winkelstein BA. Spinal PAR1 mRNA Levels are Regulated by Mechanical & Chemical Cues in Painful Nerve Root Compression. *ASME Summer Bioengineering Conference*, #SBC2011-53084, Nemacolin, PA, June 2011.

Proceedings of the ASME 2011 Summer Bioengineering Conference
SBC2011
June 22-25, Nemacolin Woodlands Resort, Farmington, Pennsylvania

SBC2011-53084

SPINAL PAR1 RNA LEVELS ARE REGULATED BY MECHANICAL & INFLAMMATORY CUES IN PAINFUL NERVE ROOT COMPRESSION

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Spinal PAR1 RNA Levels are Regulated by Mechanical & Inflammatory Cues in Painful Nerve Root Compression

Jenell R. Smith, Sarah M. Rothman, Paul A. Janmey, Beth A. Winkelstein
Department of Bioengineering, University of Pennsylvania, Philadelphia, PA

Introduction

- Disc herniation both imposes mechanical compression and introduces inflammatory agents to the spinal nerve roots that each have been shown to modulate pain in human clinical studies and in rat models [1-4].
- Mechanical compression of the nerve root induces neuronal release of neuropeptides in the dorsal horn of the spinal cord leading to central sensitization, glial activation, and pain, in a load-dependent manner as early as day 1 [3,5]. These responses are all enhanced with the addition of an inflammatory insult [3].
- Protease-activated receptors, such as PAR1, have been implicated in pain and inflammation and are known to be expressed on neurons and glia resident in the CNS [6-9]. Yet, the effect of nerve root mechanics and inflammation on spinal PAR1 production is unknown.

The goal of this study was to quantify spinal PAR1 mRNA expression after different types of painful nerve root injury and to determine if PAR1 production relates to injury cues and/or pain onset.

Methods

Surgical Procedures: Male Holtzman rats received either a C7 dorsal nerve root compression (10gf, n=3), a combined compression with inflammatory insult (10gf+chr, n=5), or a sham surgery with only exposure (sham, n=4) [3,4] (Fig. 1). Compression was applied by a calibrated 10gf clip for 15 minutes and assessments were made at day 1.

Fig. 1. Schematic of C7 spinal cord (SC), nerve roots, and DRGs. The location of the nerve root injury is indicated by the 10gf clip placed proximal to the DRG. Chromic gut suture (red) provides the inflammatory insult [3,4].

Behavioral Testing: To quantify behavioral sensitivity, mechanical allodynia in the ipsilateral forepaw was measured using two strengths of von Frey filaments (2g, 4g). Testing was performed prior to any surgical procedures (day 0; baseline) and on day 1 after the injury [2-4]. Mechanical allodynia data were statistically compared over time between all groups using a repeated measures ANOVA with Bonferroni correction.

Reverse Transcriptase PCR: Ipsilateral spinal cord tissue was harvested on day 1 after behavioral testing. Total RNA was isolated and PAR1 mRNA (fwd: 5'-AGGGGATGAG GAGGAGAAAA-3' and rev: 5'-CAGGGGAAGGCTGACTATGA-3') was quantified using reverse transcriptase PCR [6]. Gene expression was normalized to Cyclophilin-A for each sample and normalized to PAR1 levels in normal rats (n=2). Fold increases of normalized PAR1 mRNA were calculated using the ΔΔCt method [4]. A one-way ANOVA with Bonferroni correction was used to compare differences in PAR1 mRNA levels between groups.

Results

Behavior:

- Mechanical allodynia on day 1 was significantly elevated over baseline for both 10gf and 10gf+chr ($p<0.001$) injuries, but sham responses were unchanged.
- Although mechanical compression (10gf) elevated mechanical allodynia over sham responses in the ipsilateral forepaw, the increase was not significant (Fig. 2). Only the combined mechanical and inflammatory insult (10gf+chr) significantly increased allodynia over sham on day 1 for testing with both filament strengths (2g $p=0.006$; 4g $p=0.003$) (Fig. 2).

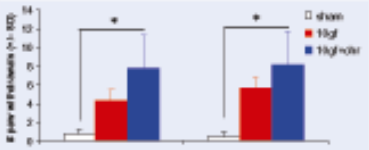

Fig. 2. Ipsilateral mechanical allodynia for 10gf+chr was significantly higher (*p<0.01) than sham on day 1, for testing with both filament strengths (2g, 4g).

Spinal PAR1 mRNA Levels:

- PAR1 mRNA was detected in all ipsilateral spinal cord samples from all groups on day 1 (Fig. 3).
- For 10gf+chr, PAR1 mRNA levels were 71.4 +/- 5.1% of those expressed in the spinal cord of normal unoperated rats. Spinal PAR1 mRNA expression for the combined injury (10gf+chr) was lower than both 10gf and sham (Fig. 3), but this decrease was only significant when compared to compression alone (10gf) ($p=0.037$).

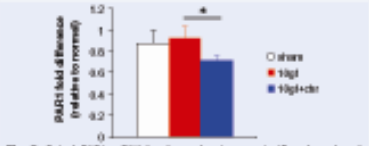

Fig. 3. Spinal PAR1 mRNA levels on day 1 were significantly reduced (*) for 10gf+chr compared to 10gf. Data are shown as mean +/- SD.

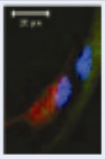

Fig. 4. PAR1 expression (green) in cultured astrocytes (red; GFAP) and neurons (unlabeled). Blue is DAPI.

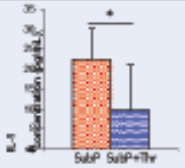

Fig. 5. SubP stimulation of rat cortical cultures. Released IL-1β was significantly reduced (*) after thrombin (Thr) treatment.

- Primary cultures of rat cortical astrocytes (GFAP) and neurons were fluorescently immunolabelled, confirming their expression of PAR1 (Fig. 4).
- Mixed cortical cultures were stimulated with substance P (SubP, n=12) to mimic a pain response in the CNS [10], and a subset was treated with the PAR1 activator, thrombin (SubP+Thr, n=6), at 24 hours later.
- Activation of PAR1 (SubP+Thr) in mixed cultures significantly decreased ($p=0.02$) release of the pro-inflammatory cytokine, IL-1β, at 24 hours compared to untreated cultures (SubP) (Fig. 5).

Discussion

- This study shows that specific injury cues modulate spinal PAR1 mRNA levels as early as day 1 after nerve root compression. Since the two injury groups (10gf & 10gf+chr) did not induce different allodynia responses (Fig. 2) but did exhibit significant differences in spinal PAR1 mRNA (Fig. 3), PAR1 production might not be regulated by the mechanical components of an injury, but may depend more on the inflammatory insult.
- The load threshold to induce the onset of pain at day 1 in this model is 26.3mN [5]. The 10gf (98.2mN) compression magnitude that was applied to the nerve root in this study is well-above that mechanical threshold. Because nerve root compression alone did not change spinal PAR1 mRNA expression (Fig. 3), these results suggest that changes in spinal PAR1 production may not result from mechanical insults or directly contribute to the onset of mechanically-induced pain.
- These data further show that the activation of PAR1 on CNS cells *in vitro* (Fig. 4) leads to a significant decrease in their release of IL-1β compared to untreated controls (Fig. 5). This confirms that PAR1 plays a role in neuroinflammation. Since spinal IL-1β is upregulated at day 1 after 10gf+chr [11], these pilot results suggest that PAR1 might contribute to early spinal inflammation; however, the mechanisms and their relationships to pain are still not fully understood.
- Although PAR1 mRNA was quantified, this study did not measure spinal PAR1 protein and only probed mRNA levels at day 1. Spinal PAR1 production may be modulated at later time points since these injuries produce time-dependent cellular and biochemical responses, including axonal degeneration that is not observed until day 7 after compression [12]. Defining temporal protein expression and mRNA patterns of PARs is necessary to fully understand these receptors' roles in pain.
- Future studies measuring PAR1 levels throughout the nervous system after injury will provide more information about whether mechanics alters PAR1 production in compressed tissue and how it relates to pain.

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Acknowledgements

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Smith JR, Weisshaar CL, Janmey PJ, Winkelstein BA. Salmon Thrombin Treatment Reduces Protease Activated Receptor 1 Expression Following Painful Nerve Root Injury. *Military Health System Research Symposium*, #12-141, Ft. Lauderdale, FL, August 2012.

SALMON THROMBIN TREATMENT REDUCES PROTEASE ACTIVATED RECEPTOR 1 EXPRESSION FOLLOWING PAINFUL NERVE ROOT INJURY

JR Smith, CL Weisshaar, PA Janmey, BA Winkelstein

PURPOSE: Protease activated receptor 1 (PAR1) cleavage by thrombin has been shown to both attenuate and exacerbate pain depending on the level of activation and its route of administration. In contrast to the generally pro-inflammatory properties of mammalian thrombin, salmon thrombin has been shown to attenuate pain after cervical nerve root compression if given at the site of injury. Although PAR1 is implicated in pain, few studies have examined its expression following injury. The purpose of this work was to define the expression of PAR1 after painful nerve root injury and to define if salmon thrombin's analgesic effect is related to PAR1.

DESIGN: Immediately following cervical nerve root compression, salmon thrombin or vehicle treatment was applied to the location of injury and rats were monitored for behavioral hypersensitivity for up to 7 days after injury. PAR1 protein expression was measured at day 1 and day 7 in both the injured nerve root and spinal cord.

POPULATION STUDIED: Male Holtzman rats (n=17; 330-440g) were used under IACUC-approved conditions.

METHOD(S): Rats underwent a C7 nerve root compression using a calibrated 10gf clip for 15 minutes; immediately after that, either salmon thrombin (0.4 U/rat) or vehicle treatment was administered at the injury site. Mechanical allodynia was measured before injury and on each postoperative day by stimulating the ipsilateral forepaw using von Frey filaments. Nerve root and spinal cord was harvested on either day 1 or 7 in separate groups, and fixed and immunolabeled for PAR1 expression.

DATA ANALYSIS: The number of paw withdrawals elicited on each day was averaged within groups and compared using a repeated measures ANOVA with a Tukey's test. PAR1 expression was quantified in the nerve root and spinal cord as a fold increase over levels in normal rats. Differences between groups were detected by a two-way ANOVA with Tukey's HSD test.

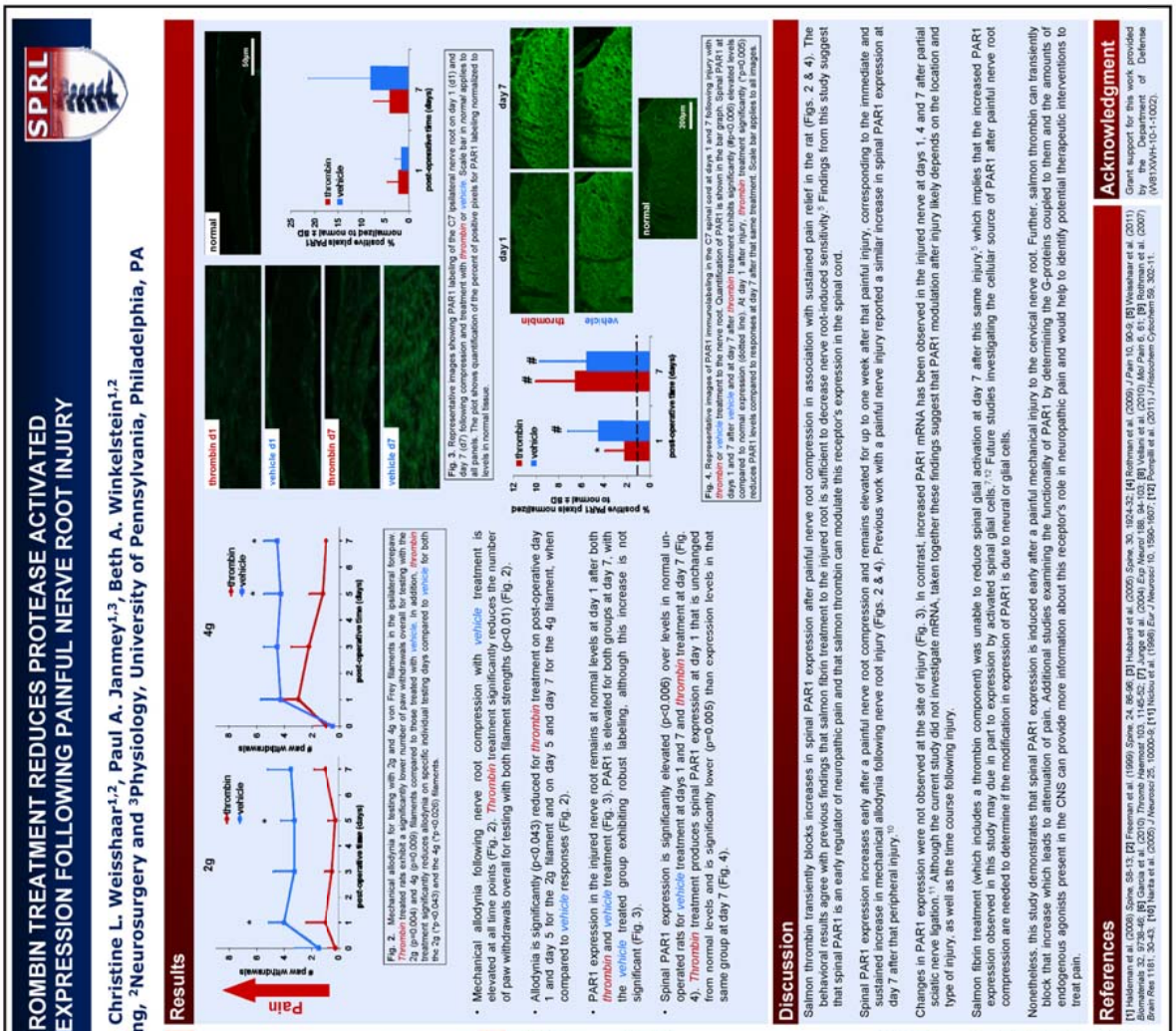
FINDINGS: Pain was significantly attenuated for rats given thrombin compared to vehicle treated rats for all days following injury ($p<0.02$). Spinal PAR1 expression was increased over normal levels for the injured groups at both time points ($p<0.01$). However, at day 1 after thrombin treatment, spinal PAR1 was not different from normal levels and was significantly lower than the expression levels at day 7 ($p=0.005$). PAR1 expression in the nerve root was not different between any groups.

CONCLUSIONS: Salmon thrombin transiently blocks PAR1 increases in the spinal cord after painful nerve root compression and provides sustained pain relief in the rat.

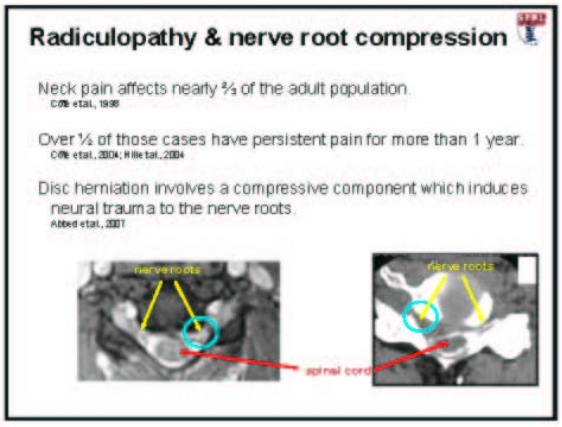
IMPLICATIONS: Increases in neural PAR1 may be linked to pain from nerve root compression and can be partially blocked by PAR1-reaction with salmon thrombin. Future studies aim to map the functionality of PAR1 by defining which cells are responsible for the increased expression and how this leads to pain.

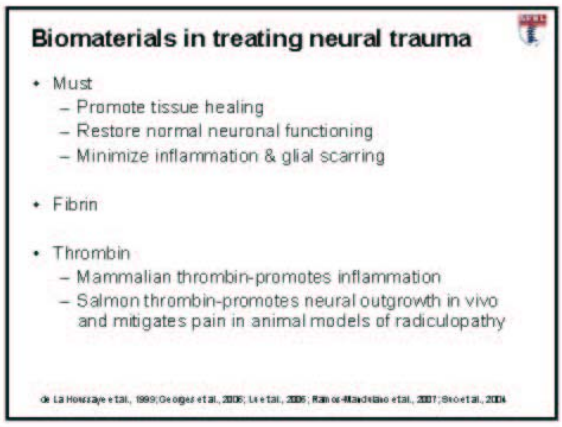
FUNDING: Support provided by the Department of Defense (W81XWH-10-1-1002).

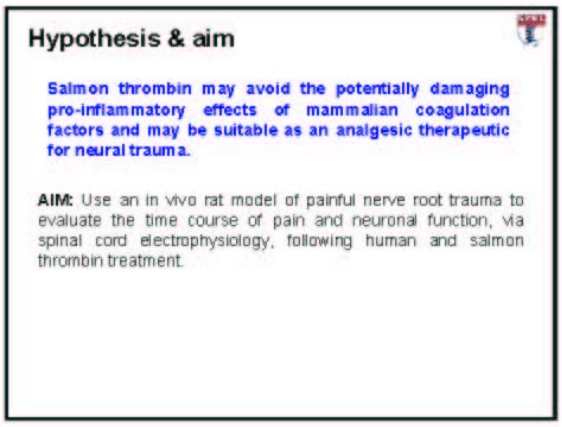
Smith JR, Weisshaar CL, Janmey PJ, Winkelstein BA. Salmon Thrombin Treatment Reduces Protease Activated Receptor 1 Expression Following Painful Nerve Root Injury. *Military Health System Research Symposium*, #12-141, Ft. Lauderdale, FL, August 2012.

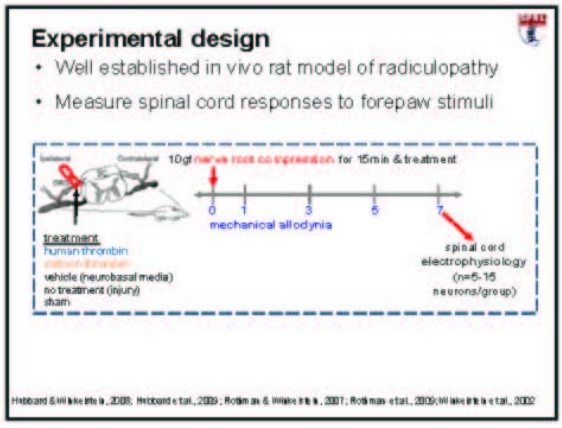


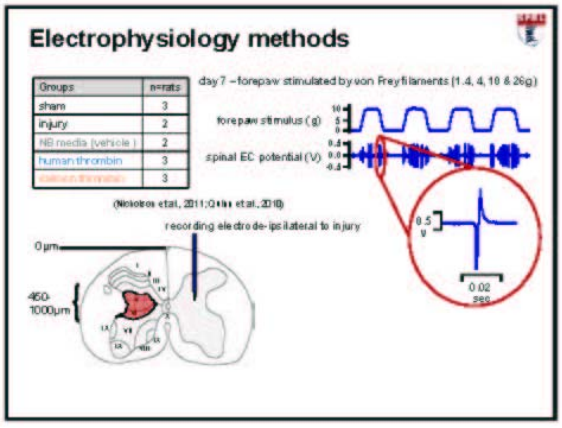
Syré P, Smith JR, Nicholson KJ, Welch WC, Janmey PA, Winkelstein BA. Salmon Thrombin Leads to Decreased Spinal Cord Responsiveness in Painful Radiculopathy. *24th Annual Pan Philadelphia Neurosurgery Conference*, Philadelphia, PA, December 2012.



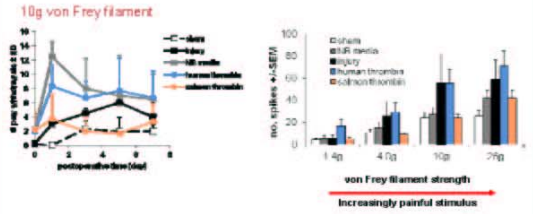








Modulation by thrombin



Conclusions & next steps

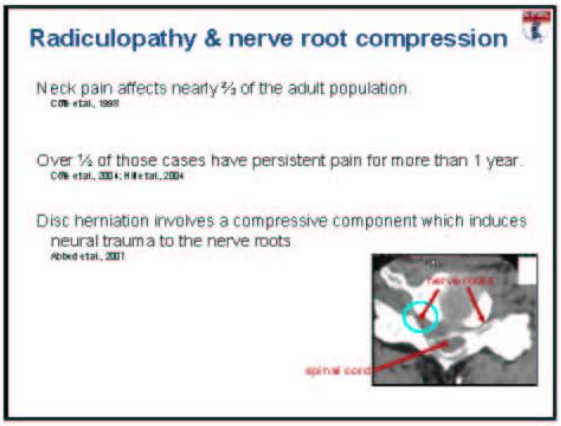
- Treatment of compressed neural tissue with salmon thrombin attenuates the behavior and spinal cord hyperactivity that is associated with a painful compressive injury or following treatment with human thrombin.
- This restored neuronal signaling may account for part of salmon thrombin's mechanism of analgesia.
- Complete further electrophysiological studies
  - Define responses to injury in the deep brain structures, such as the thalamus.

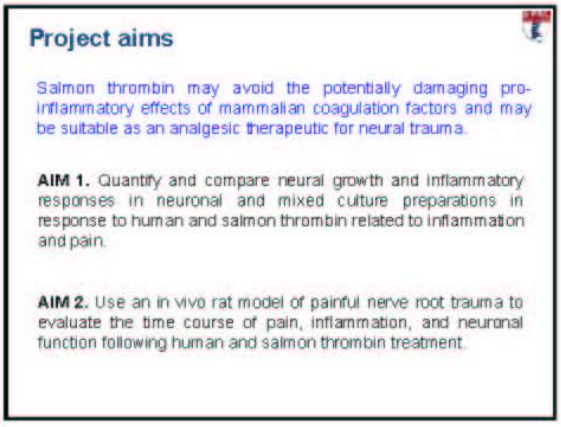
Thank you

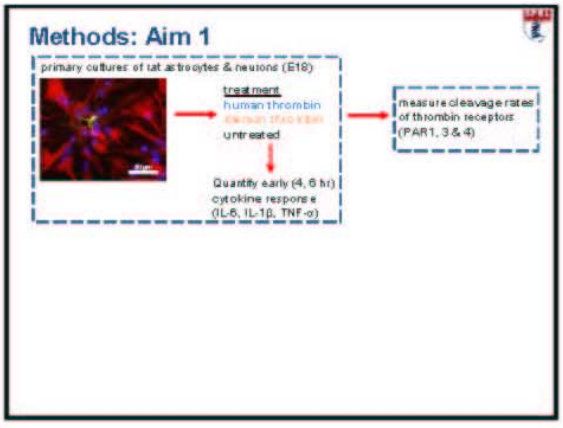
Department of Neurosurgery

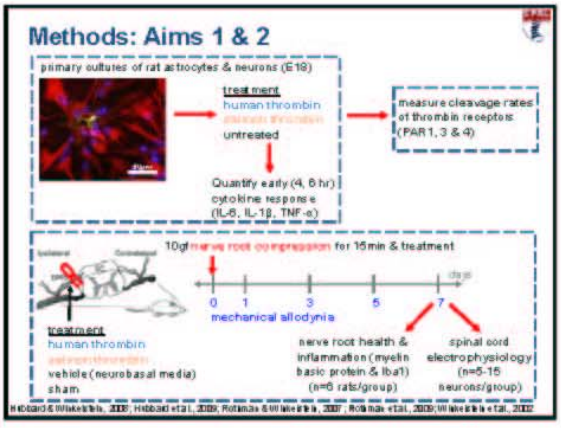
SPRL

Smith JR, Nicholson KJ, Syré P, Janmey PA, Winkelstein BA. A Novel Bioengineered Biomaterial to Treat Painful Neural Trauma via Modified Thrombin Activity for Improving Neuronal Function and Treating Pain. *Cervical Spine Research Society Annual Meeting*, Chicago, IL, December 2012.

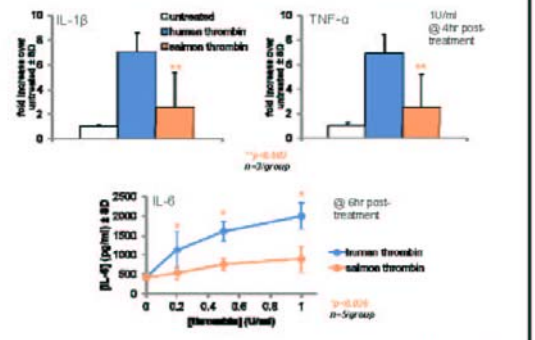






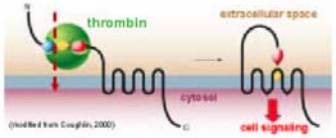


Thrombin-induced inflammation *in vitro*



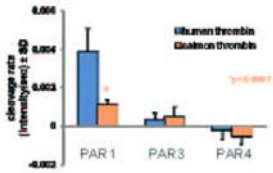
Smith et al., *under review*

PAR cleavage rate by thrombin

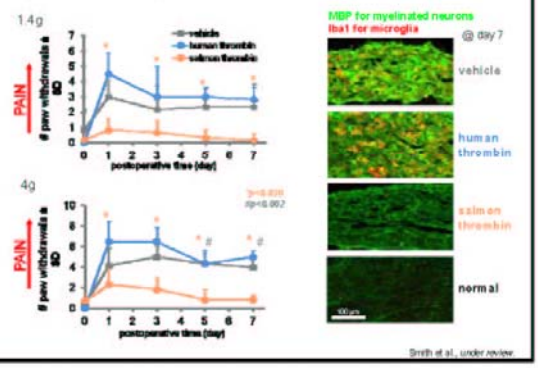


PAR peptide cleavage

PAR1	Asp-Pro-Arg-AMC
PAR3	Pro-Ile-Lys-AMC
PAR4	Ala-Pro-Arg-AMC



Myelin basic protein & Iba1 in nerve root

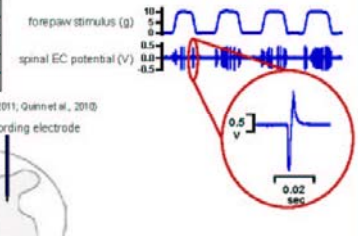


Smith et al., *under review*

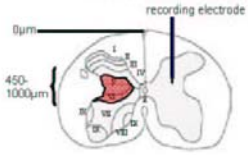
Aim 2: Electrophysiology methods

Groups	n=rats
sham	3
injury	2
vehicle	2
human thrombin	3
salmon thrombin	3

day 7 – forepaw stimulated by von Frey filaments (1.4, 4, 10 & 26g)



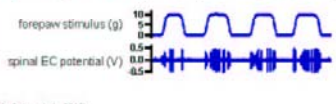
(Nicholson et al., 2011; Quinn et al., 2010)



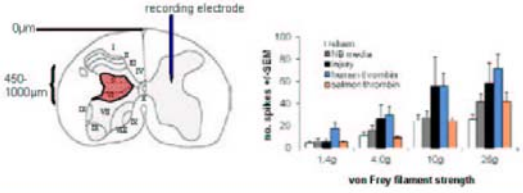
Aim 2: Electrophysiology results

Groups	n=rats
sham	3
injury	2
vehicle	2
human thrombin	3
salmon thrombin	3

day 7 – forepaw stimulated by von Frey filaments (1.4, 4, 10 & 26g)



(Nicholson et al., 2011; Quinn et al., 2010)



Ongoing activities

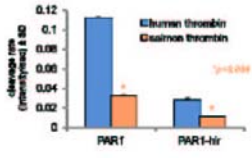
Complete electrophysiology experiments.

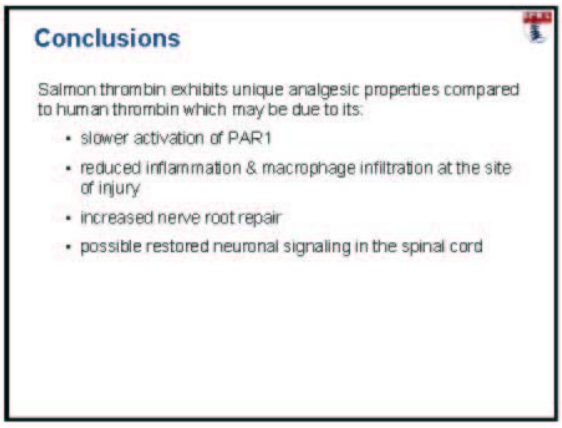
Define differences in the neural recovery associated with pain responses following human and salmon thrombin thrombin treatment.

Characterize effects on inflammatory responses in vivo & in vitro.

PAR1 kinetics:

- modified peptides
- on cells




Conclusions
Salmon thrombin exhibits unique analgesic properties compared to human thrombin which may be due to its:
• slower activation of PAR1
• reduced inflammation & macrophage infiltration at the site of injury
• increased nerve root repair
• possible restored neuronal signaling in the spinal cord